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Prenatal Exposure to Glyphosate and Its Environmental Degradate, Aminomethylphosphonic Acid (AMPA), and Preterm Birth: A Nested Case–Control Study in the PROTECT Cohort (Puerto Rico)

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Background: Glyphosate (GLY) is the most heavily used herbicide in the world. Despite nearly ubiquitous exposure, few studies have examined prenatal GLY exposure and potentially adverse pregnancy outcomes. Preterm birth (PTB) is a risk factor for neonatal mortality and adverse health effects in childhood.

Objectives: We examined prenatal exposure to GLY and a highly persistent environmental degradate of GLY, aminomethylphosphonic acid (AMPA), and odds of PTB in a nested case–control study within the ongoing Puerto Rico Testsite for Exploring Contamination Threats (PROTECT) pregnancy cohort in northern Puerto Rico.

Methods: GLY and AMPA in urine samples collected at 18 ± 2 (Visit 1) and 26 ± 2 (Visit 3) wk gestation (53 cases/194 randomly selected controls) were measured using gas chromatography tandem mass spectrometry. Multivariable logistic regression was used to estimate associations with PTB (delivery <37 wk completed gestation).

Results: Detection rates in controls were 77.4% and 77.5% for GLY and 52.8% and 47.7% for AMPA, and geometric means (geometric standard deviations) were 0.44 (2.50) and 0.41 (2.56) μg/L for GLY and 0.25 (3.06) and 0.20 (2.87) μg/L for AMPA, for Visits 1 and 3, respectively. PTB was significantly associated with specific gravity–corrected urinary GLY and AMPA at Visit 3, whereas associations with levels at Visit 1 and the Visits 1–3 average were largely null or inconsistent. Adjusted odds ratios (ORs) for an interquartile range increase in exposure at Visit 3 were 1.35 (95% CI: 0.99, 1.83) and 1.67 (95% CI: 1.26, 2.20) for GLY and AMPA, respectively. ORs for Visit 1 and the visit average were closer to the null.

Discussion: Urine GLY and AMPA levels in samples collected near the 26th week of pregnancy were associated with increased odds of PTB in this modestly sized nested case–control study. Given the widespread use of GLY, multiple potential sources of AMPA, and AMPA's persistence in the environment, as well as the potential for long-term adverse health effects in preterm infants, further investigation in other populations is warranted. https://doi.org/10.1289/EHP7295

Introduction

Glyphosate (GLY) is a broad spectrum herbicide and is the active ingredient in Roundup®, the most heavily used herbicide in the world (Benbrook 2016; Duke and Powles 2008; Woodburn 2000). GLY-based herbicides (GBHs) were first introduced to the market in mid-1970s and are still used in a wide variety of agricultural and residential applications today (Gillezeau et al. 2019). In the United States alone, over 1.6 billion kg of GLY have been applied in the last 40 y (Benbrook 2016). Over 90% of corn, soy, and canola grown in the United States is genetically modified to be GLY resistant (Fernandez-Cornejo et al. 2014), and the market is saturated with more than 750 GBH products (Landrigan and Belpoggi 2018). Long-term and widespread use has yielded detectable GLY residues in foodstuffs, soil, house dust, air, and water (Coupe et al. 2012; Curwin et al. 2005; Mercurio et al. 2014; Silva et al. 2018; Simonetti et al. 2015; USGS 2019). Therefore, nonoccupational exposure may occur through a variety of pathways including diet, drinking water, and residential use (U.S. EPA 2017).

GLY is largely not metabolized in mammals, enabling the parent compound to be measured in the urine. A study in rats showed little to no evidence of GLY metabolism, with nearly 100% of the parent compound recovered with no significant persistence of material (Brewster et al. 1991). Information on the half-life and excretion rates of GLY in humans is inconsistent (Connolly et al. 2019). Findings from animal toxicological studies extrapolated to humans suggest a first phase half-life of ∼6 h (Williams et al. 2000) and an elimination half-life of ∼33 h (IARC 2016). A recent human study, using three different metrics for estimating half-life, suggested a range of 3.5–14.5 h, with the most stable method yielding a half-life of 7.25 h [95% confidence interval (CI): 5.5, 9 h] (Connolly et al. 2019).

GLY biodegradation in the environment occurs via two primary pathways. GLY is broken down into either aminomethylphosphonic acid (AMPA) and glyoxylate by glyphosate oxidoreductase, or into sarcosine and glycine by carbon–phosphorus (C-P) lyase (Grandcoin et al. 2017). GLY is easily converted to AMPA and glyoxylate by soil microbes (Borggaard 2011; Kanissery et al. 2015), whereas GLY chemical degradation and photodegradation are minimal under natural conditions (Grandcoin et al. 2017).

In addition to being an environmental degradate of GLY, AMPA is also a breakdown product for amino-polyphosphonate chemicals, which may be used as detergents, antiscaling agents, and fire retardants (Grandcoin et al. 2017). The main phosphonate degradation pathway, under natural conditions, is metal-catalyzed photodegradation via iron–phosphonate complexes. AMPA can then be further broken down into phosphate by C-P lyase (Grandcoin et al. 2017).

Unlike GLY, which has a half-life in soil of 5–23 d (PPDB 2013b), AMPA is highly persistent in soil, with an average half-

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life of 151 d (76–240 d) (PPDB 2013a). We were unable to find any information about the half-life of AMPA in humans; however, a toxicology study in rats suggests that AMPA may be eliminated at a similar rate to GLY (Anadón et al. 2009).

In plants, GLY competitively inhibits the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), which is involved in synthesizing amino acids important for plant growth via the Shikimate pathway, causing a reduction in protein synthesis and ultimately death (Duke 2018). EPSPS is not expressed in humans or other vertebrates, rendering GLY essentially nontoxic via this mechanism in these species (Duke 2018). However, there is mounting evidence that GLY may be negatively associated with human health via other mechanisms or pathways. A 13-wk pilot study indicated that reproductive parameters (Manservisi et al. 2019) and gut microbiota (Mao et al. 2018) differed between rats exposed to 1.75 mg/kg body weight per day GBH, which is the acceptable daily intake for GLY in the United States (U.S. EPA 1993), and unexposed controls. Experimental studies have also reported birth defects in frog and chicken embryos incubated with GBH (Paganelli et al. 2010), skeletal alterations in fetuses of rats exposed to 500–1,000 mg/kg GLY during Days 6–15 of pregnancy (Dallegrave et al. 2003), and postimplantation loss, late embryonic death, pregnancy loss, and other adverse outcomes in rats, mice, and rabbits exposed to GBHs during pregnancy, as reviewed by Antoniou et al. (2012). Chromosomal and DNA damage was associated with intraperitoneal GLY injection in mice and *in vitro* exposure of cultured human lymphocytes (Bolognesi et al. 1997) and with *in vitro* endocrine disruption and cytotoxicity in human liver (Gasnier et al. 2009), placenta (Richard et al. 2005), and embryonic (Benachour et al. 2007) cell lines.

In vitro AMPA at 0.25–2.0 mM concentrations or higher induced hemolysis, decreased oxygen binding, and increased reactive oxygen species in human erythrocytes (Kwiatkowska et al. 2014); had mutagenic effects in human lymphocytes; caused DNA damage in human epithelial cells (Mañas et al. 2009); and caused cell membrane damage and cytotoxicity in primary cultured human umbilical cells and in embryonic kidney and placental cell lines (Benachour and Séralini 2009).

Despite toxicological evidence and concerns about potential negative health effects associated with GLY and AMPA exposure, few studies have examined reproductive or developmental end points in humans, such as preterm birth (PTB). PTB is defined as delivery before 37 wk completed gestation and is an immense public health problem throughout the world. PTB is one of the most important risk factors for neonatal mortality and increases the risk of comorbidities and other adverse health effects in childhood and later in life (Behrman and Butler 2007; Blencowe et al. 2013). Medical advances have greatly improved the survival rates infants born preterm, yet the long-term health and economic consequences of PTB remain a significant problem (Blencowe et al. 2013). The etiology of PTB is multifactorial, making the identification of potential causes challenging (Behrman and Butler 2007).

The United States has some of the highest rates of PTB in the developed world, and rates are particularly high on the island of Puerto Rico (March of Dimes 2010, 2011). In 2017, 11.5% of all births in Puerto Rico were preterm, earning them a grade of F on the March of Dimes "Premature Birth Report Card" for the United States that year (March of Dimes 2017). The cause(s) of the elevated rates of PTB in Puerto Rico are unknown, although high levels of environmental contaminants may play a role. There is evidence of widespread contamination in Puerto Rico; contaminants may leach into groundwater, which, in turn, feeds the karst aquifer system that supplies drinking water to the island (Padilla et al. 2011). In 2011, the Puerto Rico Testsite for Exploring Contamination Threats (PROTECT) birth cohort was established to explore the potential role of environmental toxicants, such as heavily used pesticides like GLY, in the etiology of PTB.

Several epidemiological studies have investigated associations between maternal GLY exposure and adverse pregnancy outcomes with mixed results, although only one used a GLY biomarker to classify exposure. GLY applied within 1 km of the maternal residence, as determined by agricultural pesticide use reports closest to the year of conception, was associated with increased risk of neural tube defects ($n = 731$ cases, 940 controls; 45 cases, 33 controls GLY-exposed) (Rull et al. 2006). A study of farmworker families found that GLY exposure within the 3 months prior to conception, as determined by a pesticide use questionnaire, was associated with an increased risk of spontaneous abortion at 12–19 wk gestation ($n = 3{,}936$ pregnancies, 395 spontaneous abortions; 33 cases GLY-exposed) (Arbuckle et al. 2001). A Colombian cohort study reported regional differences in time to pregnancy (TTP) among geographical regions with exposure to aerial GLY spraying, compared with a control region, although the differences in TTP were not clearly or consistently associated with GLY spraying ($n = 2{,}592$) (Sanin et al. 2009). Women enrolled in the Agricultural Health Study reported no statistically significant associations between any self-reported GLY use during pregnancy and infant birth weight ($n = 2{,}220$; 700 GLY-exposed) (Sathyanarayana et al. 2010). Finally, a cohort study in Indiana found that specific gravity–corrected GLY, measured in urine collected between 11 and 38 wk of gestation, was significantly correlated with shorter length of gestation but not with fetal growth parameters such as birth weight percentile and head circumference ($n = 71$) (Parvez et al. 2018). To our knowledge, there have been no studies to date that have examined AMPA and pregnancy-related outcomes.

Given the widespread use of GLY, mounting concerns from the toxicological literature, the methodological limitations of the currently available epidemiological literature, as well as a dearth of information about the potential health effects of AMPA, further investigation of potential adverse pregnancy outcomes following prenatal GLY and AMPA exposure is warranted. The goal of the present study was to determine the extent to which prenatal GLY and AMPA exposure is associated with PTB.

Methods

Study Population

The PROTECT study has been described in detail elsewhere (Ferguson et al. 2019a). Briefly, pregnant women, 18–40 years of age, were recruited into the PROTECT study early in pregnancy (at $\sim$14 wk of gestation) from two hospitals and five clinics in the Northern Karst aquifer region of Puerto Rico. Eligible women must have been current residents of the Northern Karst aquifer region at the time of recruitment; not have used oral contraceptives within the 3 months prior to pregnancy; not have used *in vitro* fertilization to become pregnant; and not have had any major preexisting medical or obstetric conditions, including diabetes, hypertension, or liver, kidney or cardiovascular disease. Women provided written informed consent prior to enrollment in the cohort, and the research protocols were approved by the ethics and research committees at the University of Puerto Rico and participating clinics, as well as at the University of Michigan and Northeastern University.

Last menstrual period (LMP) and demographic information were obtained at the initial screening (14 wk gestation). Spot urine samples, pregnancy characteristics, and additional demographics were obtained at three subsequent study visits, at 18 ± 2 (Visit 1), 22 ± 2 (Visit 2), and 26 ± 2 (Visit 3) wk of gestation.

At the time the present nested case–control study was designed, all preterm birth cases recorded to date who had urine samples at Visit 1 or Visit 3 available were included, along with

randomly selected controls who also had Visit 1 or Visit 3 urine samples available. The number of controls that were included was based on the resources available for biomarker measurements at the time of case–control selection.

Determination of GLY and AMPA

The analysis of urine for GLY and AMPA concentrations was performed at NSF International using gas chromatography tandem triple quadrupole mass spectrometry (GC-MS/MS) on an Agilent 7890B gas chromatograph coupled to an Agilent 7000C triple quadrupole mass spectrometer. An in-house method was developed and validated based on a method previously described by Conrad et al. (2017).

All reagents were analytical grade unless stated otherwise. Reference compounds GLY and AMPA and the derivatizing reagents trifluoroacetic anhydride (TFAA) and 2,2,2-trifluoroethanol (TFE) were purchased from Sigma-Aldrich. Internal standards (ISs) glyphosate-2-^{13}C, ^{15}N, and AMPA-^{13}CAMPA^{15}N-D_2 were obtained from Cambridge Isotope Laboratories as solutions in water. Liquid chromatography-MS–grade water was obtained from Fisher Scientific. The analytical solvents methanol, acetonitrile, and ethyl acetate were purchased from Avantor.

The unpreserved urine samples, calibration standards, matrix spike samples, and blanks were derivatized using the following method. Fifty microliters of the urine sample and 20 μL of the IS solution (50 ng/mL of each IS) were evaporated to dryness under nitrogen with 0.5 mL of acetonitrile. The derivatizing reagents TFAA (0.7 mL) and TFE (0.3 mL) were added to the samples and heated at 110°C for 1 h. After cooling to room temperature, the reaction mixture was evaporated under nitrogen and the residue was dissolved in 0.1 mL of ethyl acetate and transferred to a sample vial for GC-M/MS analysis. Calibration standards for GLY and AMPA were prepared at concentrations ranging from 0.1 to 20 ng/mL in water with 10% methanol. Matrix spikes were prepared in duplicate at analyte concentrations of 0.5 ng/mL, 2 ng/mL, and 5 ng/mL using pooled reference urine purchased from BioIVT.

The derivatized analytes were separated by GC using an Agilent 7890B gas chromatograph equipped with a Multimode Inlet injector. The GC column was an Agilent DB-624 Ultra Inert (30 m × 0.25 mm × 1.4 μm) with a 10-m guard column installed. The injector temperature was 250°C, and the injection volume was 2 μL, with pulsed splitless mode at a 344.5 kPa pulse pressure. The oven temperature was held at 80°C for 1 min, then ramped to 160°C at 20°C per min, held for 1 min, ramped at 25°C to 260°C, and held for 5 min to bake out. Helium (He) was used as the carrier gas, with a constant flow rate of 1 mL/min.

Each batch run contained a minimum of five calibration standards and duplicate QC samples of three concentrations within the established calibration range, or a minimum of 5% of the total number of samples within the batch run. Calibration curves had an R^2 of ≥0.98, and the allowed percentage deviation from nominal quality control (QC) values at all concentrations was ±15% or ±2 standard deviations (SDs) of the mean of each QC level from prior QC data. The batch run was accepted if at least 67% of the total number QC samples within a concentration met the acceptance criteria. If >2 QC samples were analyzed per concentration, 50% of the QC samples at each concentration must have met the acceptance criteria.

Quantitation was performed by an Agilent 7000C GC-MS/MS operated in multiple reaction monitoring (MRM) mode by negative chemical ionization using methane as the ionization gas. The temperatures of the ion source were 150°C, the transfer line was 260°C, and the quadrupoles were 150°C. The collision gas was nitrogen gas (N_2) at 1.5 mL/min, and the quench gas was He at 2.25 mL/min. The MRM transitions used are listed in Table S1. The first transitions were used for quantitation, and the second and third transitions were used for qualifiers.

We measured specific gravity to account for urine dilution. Specific gravity was quantified at the time of urine sample aliquoting using a digital handheld refractometer (ATAGO Co., Ltd.).

Determination of PTB

Gestational age was estimated using the American College of Obstetricians and Gynecologists (ACOG) guidelines, which are recommended as the best obstetrical estimate of gestational age. This method uses the LMP date as reported by the mother with verification by ultrasound prior to 20 wk gestation, when available. The selection of the best estimate (LMP or ultrasound) depends on both the timing of the ultrasound and the size of the difference in the estimated date of delivery that is calculated from both estimates (ACOG 2017). These methods have been well characterized and are described in detail for this cohort elsewhere (Aker et al. 2019; Ashrap et al. 2020; Ferguson et al. 2019a, 2019b). Briefly, early pregnancy ultrasounds were collected at a median of 8.4 wk gestation; ultrasound estimates were available for ∼75% of study participants. Per ACOG guidelines, LMP estimates were replaced with ultrasound estimates if the difference between the two was >5 d (for ultrasound measurements taken at <9 wk gestation) or 7 d (for ultrasound measurements taken at <14 wk gestation) (ACOG 2017). Gestational age estimates by LMP vs. ultrasound were highly correlated in the overall sample ($\rho = 0.92$, $p < 0.001$), and in the subset (17%), where estimates were changed from LMP dating to ultrasound ($\rho = 0.63$, $p < 0.001$). PTB was defined as delivery before 37 completed wk of gestation. Spontaneous PTB was defined as PTB with premature rupture of the membranes, spontaneous preterm labor, or both (Ferguson et al. 2014). PTB with preeclampsia or with artificial membrane rupture and induced labor were classified as nonspontaneous PTB (Ferguson et al. 2014).

Statistical Analysis

Statistical analyses were conducted using SAS (version 9.4; SAS Institute, Inc.). Descriptive statistics and frequencies were examined for all variables of interest. To account for urine dilution, we corrected for specific gravity using the following formula: $GA_{SG} = GA[(1.019–1)/(SG–1)]$, where GA_{SG} is the specific gravity–corrected GLY or AMPA concentration (in micrograms per liter); GA is the observed GLY or AMPA concentration; 1.019 is the population median specific gravity; and SG is the specific gravity of the urine sample. Percentile tables were created to determine the exposure profiles for GLY_{SG} and $AMPA_{SG}$ in our sample; arithmetic and geometric means (GMs) were also calculated. Spearman correlation coefficients were calculated to examine relationships between GLY_{SG} and $AMPA_{SG}$ at both visits. To assess the ratio of within- to between-individual variability in GLY_{SG} and $AMPA_{SG}$ over pregnancy, we calculated intraclass correlation coefficients (ICCs) for individuals with measurements at both time points (Rosner 2000). To create a more stable estimate of individual exposure during pregnancy, we created subject-specific pregnancy averages for GLY_{SG} and $AMPA_{SG}$ by averaging the specific gravity–adjusted values at Visits 1 and 3.

GLY and AMPA values below the limit of detection (<LOD) were replaced with the LOD divided by the square root of 2 if no value was reported; if a value was reported, the reported value was used in place of the LOD divided by the square root of 2. Multivariable logistic regression was used to calculate associations between an interquartile range (IQR) difference in GLY and AMPA, at each Visit (1 and 3), as well as the average of the two visits, and PTB. IQRs were calculated based on distributions in controls only. All biomarkers were specific gravity corrected prior to inclusion in the models.

To examine trends, we created tertiles for specific gravity–corrected GLY and AMPA at each visit and for the pregnancy averages. GLY was categorized into equally sized tertiles based on distribution in the controls (low/medium/high). Due to >33% of AMPA values being <LOD, AMPA was categorized into <LOD divided by the medium/high (median split among the detects) based on the distribution in the controls. AMPA was coded into equally sized tertiles (low/medium/high) for the visit average. p_{Trend}-Values were derived by modeling integer-scored tertiles of GLY and AMPA as ordinal variables.

Confounders considered for inclusion in our adjusted models were maternal age, level of maternal education (high school or less/some college or technical school/college degree or higher), household income (<USD10,000/USD10,000–29,999/USD30,000–49,999/≥USD50,000), prepregnancy body mass index (BMI), maternal smoking (ever/never), and prior PTB (yes/no) (Figure S1). Potential covariates were entered into the model one at a time using a forward stepwise procedure and retained for those that influenced effect estimates by >10%. This procedure yielded only education and prepregnancy BMI as covariates. Based on our directed acyclic graph (Figure S1), maternal age and smoking were considered potentially important confounders and were thus included in the model. Household income and prior PTB had a relatively high percentage of missing values and were excluded from our main models but included as a sensitivity analysis. Thus, our primary models were adjusted for maternal age, education, prepregnancy BMI, and maternal smoking. All adjusted models were complete case analyses.

We completed several additional analyses to test the robustness of our results and explore additional variables of interest. As mentioned previously, we carried out sensitivity analyses to assess potential confounding by the variables that were not included in our final models by adjusting for household income and prior PTB. We additionally assessed for potential confounding by di-*n*-butyl phthalate and di-isobutyl phthalate by adding their urinary metabolites, mono-*n*-butyl phthalate (MBP) and mono-isobutyl phthalate (MiBP), to our models. MBP and MiBP have been previously found to be associated with increased odds of PTB in PROTECT (Ferguson et al. 2019b). MBP and MiBP were specific gravity corrected and natural log-transformed prior to inclusion in the models.

We also ran our logistic regression models stratified by infant sex to test for possible sex-specific effects because rodent models have revealed the potential for endocrine disruption following GLY exposure (Manservisi et al. 2019). Unstratified logistic regression models were then used to test for statistical significance of potential sex-specific effects, by adding sex and sex–GLY or sex–AMPA interaction terms to the models and examining the statistical significance of the interaction terms.

Finally, we examined two alternative outcomes of interest: spontaneous PTB and length of gestation. Spontaneous PTB was defined as premature rupture of the membranes, spontaneous preterm labor, or both, and may represent a more etiologically homogeneous subset of preterm births appropriate to explore in relation to environmental exposures (Ferguson et al. 2014). Multivariable generalized linear models were used to estimate associations between GLY and AMPA and length of gestation in weeks.

Results

247 women total had urinary GLY and AMPA measurements (53 cases, 194 controls), 177 at Visit 1 (35 cases, 142 controls), 208 at Visit 3 (53 cases, 208 controls), and 138 (35 cases, 103 controls) at both time points. By chance, all PTB cases had Visit 3 urine samples, with a subset of those also having a sample for Visit 1; there were no cases that had only a Visit 1 sample. The LOD was 0.20 μg/L for both GLY and AMPA. GLY was detected in 79.1% and 79.3% of samples, whereas AMPA was detected in 54.2% and 51.4% of samples, for Visits 1 and 3, respectively. Quality control analysis yielded coefficients of variation ranging from 7.0% to 14.4%. GMs [geometric standard deviations (GSDs)] for GLY, at Visits 1 and 3, respectively, were 0.45 (2.17) and 0.56 (2.58) μg/L for cases and 0.44 (2.50) and 0.41 (2.56) μg/L for controls (Table 1). GMs (GSDs) for AMPA, at Visits 1 and 3, respectively, were 0.24 (2.76) and 0.33 (3.40) μg/L for cases and 0.25 (3.06) and 0.20 (2.87) μg/L for

Table 1. Distribution of specific gravity–corrected GLY and AMPA in the urine (μg/L) at Visit 1 (18 ± 2 wk gestation), Visit 3 (26 ± 2 wk gestation), and the average of Visits 1 and 3 in the PROTECT cohort.

								Percentile						
Analyte	Visit	*n*	Percentage <LOD[a]	AM	SD	GM	GSD	10th	25th	Median	75th	90th	95th	Max.
Overall sample														
GLY	1	177	20.9	0.60	0.44	0.44	2.43	0.14 (<LOD)	0.28	0.50	0.79	1.15	1.55	2.80
	3	208	20.7	0.65	0.66	0.44	2.59	0.14 (<LOD)	0.27	0.47	0.82	1.34	1.71	5.36
	Avg.	138	8.6	0.61	0.41	0.49	1.98	0.22	0.31	0.50	0.82	1.07	1.48	2.73
AMPA	1	177	45.8	0.44	0.84	0.25	2.99	0.07 (<LOD)	0.14 (<LOD)	0.26	0.53	0.83	1.22	10.08
	3	208	47.6	0.38	0.41	0.23	3.05	0.06 (<LOD)	0.13 (<LOD)	0.23	0.53	0.85	1.06	3.08
	Avg.	138	29.0	0.37	0.29	0.28	2.15	0.09 (<LOD)	0.17 (<LOD)	0.30	0.47	0.71	0.86	1.85
Preterm cases														
GLY	1	35	14.3	0.60	0.52	0.45	2.17	0.11 (<LOD)	0.31	0.46	0.79	1.09	1.78	2.80
	3	53	15.1	0.86	1.02	0.56	2.58	0.18 (<LOD)	0.36	0.55	1.00	1.28	4.02	5.36
	Avg.	35	5.7	0.67	0.52	0.54	1.96	0.29	0.34	0.51	0.82	0.97	1.98	2.73
AMPA	1	35	40.0	0.35	0.30	0.24	2.76	0.08 (<LOD)	0.16 (<LOD)	0.27	0.48	0.79	1.02	1.25
	3	53	34.0	0.58	0.63	0.33	3.40	0.10 (<LOD)	0.19 (<LOD)	0.38	0.73	1.32	1.97	3.38
	Avg.	35	25.7	0.45	0.37	0.34	2.20	0.11 (<LOD)	0.20	0.36	0.59	0.88	1.31	1.80
Full-term controls														
GLY	1	142	22.5	0.60	0.43	0.44	2.50	0.14 (<LOD)	0.28	0.52	0.79	1.15	1.53	1.95
	3	155	22.6	0.58	0.47	0.41	2.56	0.13 (<LOD)	0.25	0.45	0.76	1.34	1.71	2.52
	Avg.	103	9.7	0.59	0.37	0.48	1.99	0.20	0.28	0.50	0.84	1.07	1.34	1.65
AMPA	1	142	47.2	0.46	0.93	0.25	3.06	0.07 (<LOD)	0.13 (<LOD)	0.26	0.53	0.83	1.22	10.08
	3	155	52.3	0.31	0.28	0.20	2.87	0.06 (<LOD)	0.12 (<LOD)	0.21	0.42	0.67	0.89	1.52
	Avg.	103	30.1	0.34	0.26	0.26	2.13	0.08 (<LOD)	0.16 (<LOD)	0.29	0.42	0.66	0.77	1.85

Note: AM, arithmetic mean; AMPA, aminomethylphosphonic acid; ASD, arithmetic standard deviation; avg., average; GLY, glyphosate; GM, geometric mean; GSD, geometric standard deviation; LOD, limit of detection; max., maximum; PROTECT, Puerto Rico Testsite for Exploring Contamination Threats.
[a]LOD = 0.20 μg/L.

controls (Table 1). 138 (35 PTB cases and 103 term controls) participants had urine samples that were measured for GLY and AMPA at both Visits 1 and 3. ICCs were 0.24 (95% CI: 0.10, 0.46) and 0.63 (95% CI: 0.48, 0.75) for specific gravity–corrected GLY and AMPA, respectively. Specific gravity–corrected GLY and AMPA were correlated at Visit 1 (Spearman $\rho = 0.43$, $p < 0.0001$) and Visit 3 (Spearman $\rho = 0.51$, $p < 0.0001$). GLY at Visits 1 and 3 was also correlated (Spearman $\rho = 0.36$, $p < 0.0001$), as was AMPA (Spearman $\rho = 0.19$, $p = 0.03$).

Compared with controls, cases were more likely to be younger (50.9% of cases were <25 years of age, compared with 38.7% of controls), less educated (32.7% of cases had a high school education or less, compared with 19.4% of controls), less likely to be employed (51.0% of cases were unemployed, compared with 35.9% of controls), lower income (47.8% of cases had a household income of <USD10,000, compared with 31.4% of controls), more likely to have never consumed alcohol (55.8% of cases were never-drinkers, compared with 43.5% of controls), and more likely to have had a prior PTB (20.8% of cases reported having a prior PTB, compared with 3.9% of controls) (Table 2). Specific gravity–corrected GMs for MBP and MiBP were also higher for cases, compared with controls, at Visit 3 (Table 2).

Adjusted ORs for associations with IQR increases at Visit 3 were positive for both GLY [odds ratio (OR) = 1.35 (95% CI: 0.99, 1.83)] and AMPA [OR = 1. 67 (95% CI: 1.26, 2.20)] (Table 3). ORs for IQR increases at Visit 1 and the visit average were based on fewer observations and were, therefore, less precise than corresponding estimates for Visit 3, but both were closer to the null. Adjusted ORs for an IQR increase in GLY were 1.11 (95% CI: 0.71, 1.74) and 1.06 (95% CI: 0.57, 1.96), for Visit 1 and the visit average, respectively; adjusted ORs for an IQR increase in AMPA were 0.92 (95% CI: 0.67,1.27) and 1.28 (95% CI: 0.87, 1.87), for Visit 1 and the visit average, respectively (Table 3).

When modeled as tertiles, adjusted ORs increased monotonically for both GLY and AMPA at Visit 3 ($p_{Trend} = 0.09$ and 0.006 for GLY and AMPA, respectively) (Figure 1, Table S2). Corresponding ORs for tertiles were imprecise for Visit 1 and the average of Visits 1 and 3, without significant trends or consistent patterns (Figure 1, Table S2).

Additional adjustment for household income and prior PTB yielded findings similar to those of the primary models. Adjusted ORs for associations with IQR increases at Visit 3 remained positive for both GLY [OR = 1.35 (95% CI: 0.96, 1.89)] and AMPA [OR = 1.59 (95% CI: 1.15, 2.21)], whereas adjusted ORs for Visit 1 and the visit average remained closer to the null for both GLY and AMPA (Table 4).

Additional adjustment for phthalate metabolites, either MBP or MiBP, also yielded findings similar to those of the primary models for GLY [OR = 1.36 (95% CI: 1.00, 1.86) and 1.38 (95% CI: 1.01, 1.88), for MBP and MiBP, respectively] and AMPA [OR = 1.70 (95% CI: 1.28, 2.26) and 1.69 (95% CI: 1.27, 2.25), for MBP and MiBP, respectively] at Visit 3; adjusted ORs for Visit 1 and the visit average stayed closer to the null for both GLY and AMPA (Table 4). GLY was weakly correlated with MBP and MiBP at Visit 1 [Spearman $\rho = 0.22$ ($p = 0.003$) and 0.23 ($p = 0.002$) for MBP and MiBP, respectively]; AMPA was not correlated with MBP or MiBP at Visit 1 [Spearman $\rho = 0.11$ ($p = 0.15$) and 0.10 ($p = 0.21$) for MBP and MiBP, respectively]. GLY and AMPA were also weakly correlated with MBP at Visit 3; Spearman $\rho = 0.24$ ($p = 0.0006$) and 0.26 ($p = 0.0002$) for GLY and AMPA, respectively. MBP and MiBP were moderately to strongly correlated at Visits 1 and 3; Spearman $\rho = 0.64$ ($p < 0.0001$) and 0.67 ($p < 0.0001$) for Visits 1 and 3, respectively.

There were no notable findings when primary models were stratified by infant sex for Visit 1, 3, or the visit average. Sample sizes for sex-stratified models were small and any apparent differences were not statistically significant (all $p_{Interaction} > 0.20$) (Table S3).

Of the 53 PTBs in this analysis, 35 (66%) were classified as the spontaneous subtype. Analyses of spontaneous PTB as a secondary

Table 2. Characteristics of the study sample ($n = 247$).

Characteristic	Full-term ($n = 194$)	Preterm ($n = 53$)	p-Value[a]
Maternal age at enrollment {y [n (%)]}			0.27
<25	75 (38.7)	27 (50.9)	
25–30	75 (38.7)	17 (32.1)	
>30	44 (22.7)	9 (17.0)	
Maternal education [n (%)]			0.02
High school or less	37 (19.4)	17 (32.7)	
Some college or technical school	63 (33.0)	21 (40.4)	
College or higher	91 (47.6)	14 (26.9)	
Missing	3	1	
Prepregnancy BMI {kg/m^2 [n (%)]}			0.16
≤25	87 (46.3)	26 (52.0)	
>25 to ≤30	59 (31.4)	9 (18.0)	
>30	42 (22.3)	15 (30.0)	
Missing	6	3	
Smoking status [n (%)]			0.89
Never	161 (83.9)	44 (84.6)	
Ever	24 (12.5)	6 (11.5)	
Current	7 (3.6)	2 (3.8)	
Missing	2	1	
Sex of infant [n (%)]			0.28
Female	92 (47.9)	21 (39.6)	
Male	100 (52.1)	32 (60.4)	
Missing	2	0	
Employment status [n (%)]			0.06
Employed	123 (64.1)	25 (49.0)	
Unemployed	69 (35.9)	26 (51.0)	
Missing	2	2	
Marital status [n (%)]			0.38
Single	38 (19.8)	9 (17.3)	
Married	106 (55.2)	25 (48.1)	
Cohabitating	48 (25.0)	18 (34.6)	
Missing	2	1	
Household income {USD [n (%)]}			0.11
<10,000	55 (31.4)	22 (47.8)	
10,000–29,999	53 (30.3)	14 (30.4)	
30,000–49,999	42 (24.0)	5 (10.9)	
≥50,000	25 (14.3)	5 (10.9)	
Missing	19	7	
Alcohol use [n (%)]			0.21
Never	83 (43.5)	29 (55.8)	
Before pregnancy only	97 (50.8)	22 (42.3)	
During pregnancy	11 (5.8)	1 (1.9)	
Missing	3	1	
Prior PTB [n (%)]			0.001
No	146 (96.1)	38 (79.2)	
Yes	6 (3.9)	10 (20.8)	
Missing	42	5	
Phthalate metabolite biomarkers {ng/mL [GM (GSD)]}[b]			
MBP Visit 1	12.8 (2.7)	13.7 (2.3)	0.71
Missing	0	0	
MBP Visit 3	13.3 (3.0)	20.5 (2.1)	0.002
Missing	3	1	
MiBP Visit 1	9.9 (2.4)	11.3 (3.0)	0.48
Missing	0	0	
MiBP Visit 3	9.6 (2.6)	14.0 (2.2)	0.01
Missing	3	1	

Note: BMI, body mass index; GM, geometric mean; GSD, geometric standard deviation; MBP, mono-n-butyl phthalate; MiBP, mono-isobutyl phthalate; PTB, preterm birth.
[a]p-Values are calculated from chi-squared tests (categorical variables) or t-tests (continuous variables).
[b]Phthalate metabolite biomarkers are corrected for specific gravity.

Table 3. Odds ratios for an interquartile range change in urinary GLY and AMPA concentrations during pregnancy and preterm birth.

	Crude[a]			Adjusted[b]		
Analyte	Preterm [*n*]	Term [*n*]	OR (95% CI)	Preterm [*n*]	Term [*n*]	OR (95% CI)
IQR GLY[c]						
Visits 1 and 3 average	35	103	1.29 (0.79, 2.12)	32	97	1.06 (0.57, 1.96)
Visit 1 (18 ± 2 wk)	35	142	1.01 (0.65, 1.55)	32	135	1.11 (0.71, 1.74)
Visit 3 (26 ± 2 wk)	53	155	1.38 (1.06, 1.79)	50	148	1.35 (0.99, 1.83)
IQR AMPA[d]						
Visits 1 and 3 average	35	103	1.36 (0.98, 1.89)	32	97	1.28 (0.87, 1.87)
Visit 1 (18 ± 2 wk)	35	142	0.89 (0.64, 1.25)	32	135	0.92 (0.67, 1.27)
Visit 3 (26 ± 2 wk)	53	155	1.57 (1.22, 2.02)	50	148	1.67 (1.26, 2.20)

Note: AMPA, aminomethylphosphonic acid; BMI, body mass index; CI, confidence interval; GLY, glyphosate; IQR, interquartile range; OR, odds ratio.
[a]Biomarkers are adjusted for specific gravity using the formula: $GA_{SG} = GA[(1.019–1)/(SG–1)]$, where GA_{SG} is the SG-adjusted GLY or AMPA concentration (μg/L); GA is the observed GLY or AMPA concentration; 1.019 is the population median specific gravity; and SG is the specific gravity of the urine sample.
[b]Models are adjusted for maternal age, education, prepregnancy BMI, and smoking.
[c]IQRs were calculated based on GLY distribution in controls. IQRs were 0.56, 0.52, and 0.54 for Visit 1, Visit 3, and the Visits 1 and 3 average, respectively.
[d]IQRs were calculated based on AMPA distribution in controls. IQRs were 0.27, 0.40, and 0.30 for Visit 1, Visit 3, and the Visits 1 and 3 average, respectively.

outcome yielded adjusted ORs for associations with IQR increases at Visit 3 that were positive for both GLY [OR = 1.45 (95% CI: 1.00, 2.10)] and AMPA [OR = 1.88 (95% CI: 1.35, 2.62)] (Table 5). Adjusted ORs for GLY at Visit 1 and the visit average were also positive, but closer to the null, whereas adjusted ORs for AMPA at Visit 1 and the visit average were less consistent, but also closer to the null (Table 5). When modeled as tertiles, adjusted ORs increased monotonically for both GLY and AMPA at Visit 3 ($p_{Trend} = 0.03$ and 0.002 for GLY and AMPA, respectively) (Figure 2, Table S4). Corresponding ORs for tertiles were imprecise, without significant trends or consistent patterns for GLY at Visit 1 and the visit average, but increased monotonically for AMPA at both Visit 1 ($p_{Trend} = 0.06$) and the visit average ($p_{Trend} = 0.04$) (Figure 2, Table S4).

Analyses using length of gestation as a secondary outcome yielded adjusted estimates for associations with IQR increases at Visit 3 that were negative for both GLY [β = −0.30 (95% CI: −0.63, 0.03); $p = 0.07$] and AMPA [β = −0.53 (95% CI: −0.82, −0.25); $p < 0.001$] (Table S5). Adjusted estimates for GLY at Visit 1 and the visit average were also negative, but closer to the null, whereas adjusted estimates for AMPA at Visit 1 and the visit average were less consistent, also remaining closer to the null (Table S5).

Discussion

We performed a nested case–control study of PTB in association with specific gravity–corrected urine concentrations of GLY and AMPA, an environmental degradation product of GLY, among women in northern Puerto Rico. Associations were consistently positive for both compounds in urine samples collected at 24–28 wk of gestation (Visit 3), but were inconsistent or null for samples

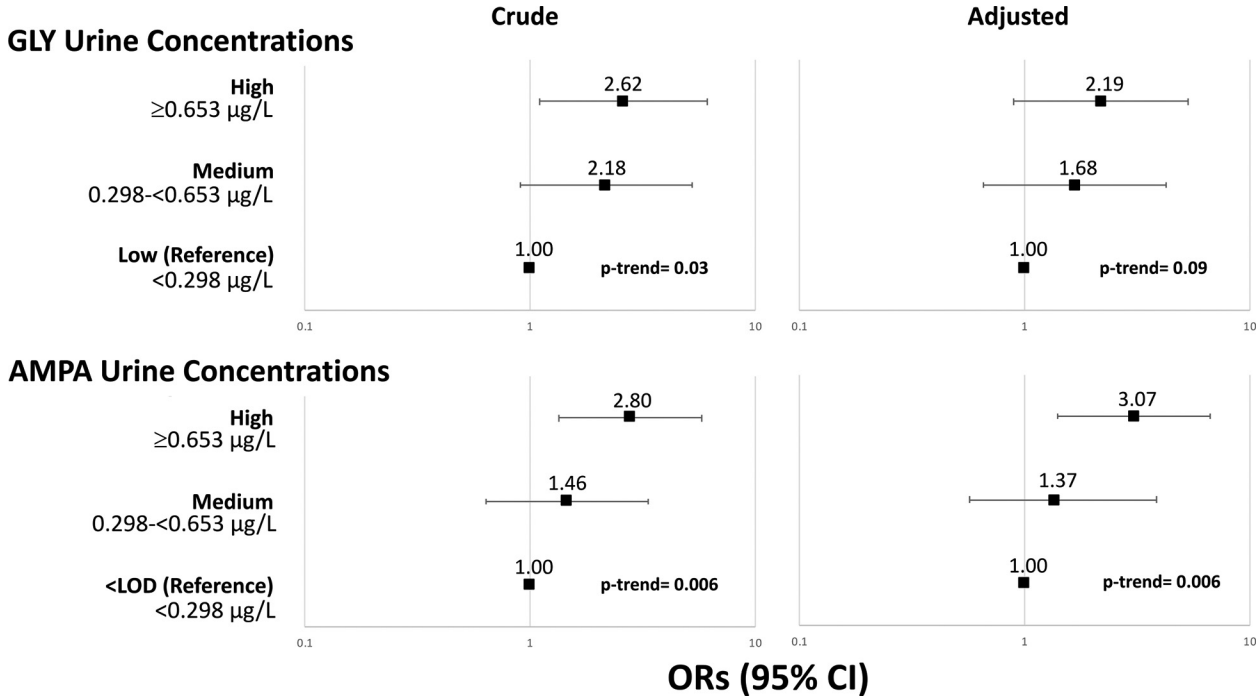

Figure 1. Odds ratios by level of urinary GLY and AMPA concentrations measured in maternal urine at Visit 3 (26 ± 2 wk gestation) and preterm birth. Corresponding numeric data for Visit 1, Visit 3, and the pregnancy average are provided in Table S3. Biomarkers are adjusted for specific gravity using the formula: $GA_{SG} = GA[(1.019–1)/(SG–1)]$, where GA_{SG} is the SG-adjusted GLY or AMPA concentration (μg/L); GA is the observed GLY or AMPA concentration; 1.019 is the population median specific gravity; and SG is the specific gravity of the urine sample; $n = 208$; 53 cases, 155 controls. Adjusted models are adjusted for maternal age, education, prepregnancy BMI, and smoking; $n = 198$; 50 cases, 148 controls. The *x*-axes are shown on a log-10 scale. Note: AMPA, aminomethylphosphonic acid; BMI, body mass index; CI, confidence interval; GLY, glyphosate; OR, odds ratio.

Table 4. Odds ratios for an interquartile range change in urinary GLY and AMPA concentrations during pregnancy and preterm birth, with adjustment for additional potential confounders.

Analyte[a]	*n* Preterm	*n* Term	OR (95% CI)
Primary model[b]			
IQR GLY[c]			
Visit 1 and 3 average	35	103	1.06 (0.57, 1.96)
Visit 1 (18 ± 2 wk)	35	142	1.11 (0.71, 1.74)
Visit 3 (26 ± 2 wk)	53	155	1.35 (0.99, 1.83)
IQR AMPA[d]			
Visit 1 and 3 average	35	103	1.28 (0.87, 1.87)
Visit 1 (18 ± 2 wk)	35	142	0.92 (0.67, 1.27)
Visit 3 (26 ± 2 wk)	53	155	1.67 (1.26, 2.20)
Primary model[b] + household income and prior PTB			
IQR GLY[c]			
Visit 1 and 3 average	26	69	0.81 (0.36, 1.80)
Visit 1 (18 ± 2 wk)	26	97	0.92 (0.51, 1.64)
Visit 3 (26 ± 2 wk)	41	105	1.35 (0.96, 1.89)
IQR AMPA[d]			
Visit 1 and 3 average	26	69	1.22 (0.73, 2.04)
Visit 1 (18 ± 2 wk)	26	97	0.85 (0.50, 1.46)
Visit 3 (26 ± 2 wk)	41	105	1.59 (1.15, 2.21)
Primary model[b] +MBP			
IQR GLY[c]			
Visit 1 and 3 average	32	95	1.03 (0.55, 1.91)
Visit 1 (18 ± 2 wk)	32	135	1.12 (0.71, 1.76)
Visit 3 (26 ± 2 wk)	49	146	1.36 (1.00, 1.86)
IQR AMPA[d]			
Visit 1 and 3 average	32	95	1.23 (0.84, 1.80)
Visit 1 (18 ± 2 wk)	32	135	0.92 (0.67, 1.27)
Visit 3 (26 ± 2 wk)	49	146	1.70 (1.28, 2.26)
Primary model[b] +MiBP			
IQR GLY[c]			
Visit 1 and 3 average	32	95	1.10 (0.59, 2.05)
Visit 1 (18 ± 2 wk)	32	135	1.04 (0.65, 1.66)
Visit 3 (26 ± 2 wk)	49	146	1.38 (1.01, 1.88)
IQR AMPA[d]			
Visit 1 and 3 average	32	95	1.27 (0.87, 1.85)
Visit 1 (18 ± 2 wk)	32	135	0.92 (0.67, 1.28)
Visit 3 (26 ± 2 wk)	49	146	1.69 (1.27, 2.25)

[a]Biomarkers are adjusted for specific gravity using the formula: $GA_{SG} = GA[(1.019–1)/(SG–1)]$ where GA_{SG} is the SG-adjusted GLY or AMPA concentration (μg/L), GA is the observed GLY or AMPA concentration, 1.019 is the population median specific gravity, and SG is the specific gravity of the urine sample.
[b]Primary model is adjusted for maternal age, education, pre-pregnancy BMI, and smoking.
[c]IQRs were calculated based on GLY distribution in controls. IQRs were 0.56, 0.52, and 0.54 for visit 1, visit 3, and the visit 1 and 3 average, respectively.
[d]IQRs were calculated based on AMPA distribution in controls. IQRs were 0.27, 0.40, and 0.30 for visit 1, visit 3, and the visit 1 and 3 average, respectively.

collected at 16–20 wk (Visit 1) and for average levels in both samples. Adjusted ORs for associations with IQR increases at Visit 3 were positive for both GLY and AMPA, while tertile analyses also yielded positive monotonic trends for both GLY and AMPA. Visit 3 results did not substantially change with the addition of household income and prior PTB or concentrations of MBP or MiBP to the models. Stratification by infant sex did not yield any notable findings for Visit 1, Visit 3, or the visit average. Findings were consistent when PTB was limited to the spontaneous PTB subtype; adjusted ORs for associations with IQR increases at Visit 3 were positive for both GLY and AMPA, and tertile analyses yielded monotonic trends for GLY and AMPA. Urinary concentrations of GLY and AMPA at Visit 3 were also associated with decreases in length of gestation; length of gestation decreased by 0.30 wk (∼2 d) and 0.53 wk (∼4 d) for an IQR increase in GLY and AMPA at Visit 3, respectively. In general, results for all models were largely null, with inconsistent trends for GLY and AMPA measured at Visit 1 and for the average of Visits 1 and 3.

The levels of GLY exposure seen in the present study are generally lower than those reported in other studies of relevant populations elsewhere. The only other study to measure GLY in the urine of pregnant women (11–38 wk gestation; $n = 71$) reported a specific gravity–corrected arithmetic mean (SD) of 3.40 (1.24) μg/L and a range 0.5–7.20 μg/L (Parvez et al. 2018). Another U.S. study reported a creatinine-corrected GM (range) of 1.2 (0.062–5.0) μg/L for GLY in the urine of mothers (with children <16 years of age) from non-farmworker families ($n = 93$ samples; 23 subjects) (Curwin et al. 2007). A Danish study of mothers (with children 6–11 years of age; $n = 13$) found a creatinine-corrected arithmetic mean (range) for GLY in urine of 1.28 (0.49–3.22) μg/L (Knudsen et al. 2017). Only one study measured both GLY and AMPA, although results did not appear to be corrected for urinary dilution; U.S. women who were 1–3 months postpartum and lactating ($n = 40$) had uncorrected arithmetic means (SDs) and ranges of 0.28 (0.38) μg/L and <0.02–1.93 μg/L for GLY in urine and 0.30 (0.33) μg/L and <0.03–1.33 μg/L for AMPA in urine (McGuire et al. 2016). With the exception of the study by McGuire et al. (2016), the measures of central tendency reported in these other studies were higher than those in the present study, where we report arithmetic means (SDs) in controls of 0.60 (0.43) μg/L and 0.58 (0.47) μg/L for GLY in urine, at Visits 1 and 3, respectively. Similarly, the maximum concentrations reported here for GLY in controls (ranges of <0.20–1.95 μg/L and <0.20–2.52 μg/L, for Visits 1 and 3, respectively) are lower than those reported in the other studies. Only the study by McGuire et al. (2016) measured AMPA, which, as noted previously, was uncorrected for urine dilution; the present study yielded a similar arithmetic mean (SD) for AMPA in the urine of controls at Visit 3 [0.31 (0.28)μg/L], but a higher arithmetic mean (SD) for AMPA in the urine of controls at Visit 1 [0.46 (0.93) μg/L]. Similarly, the maximum concentrations reported here for

Table 5. Odds ratios for an interquartile range change in urinary GLY and AMPA concentrations during pregnancy and spontaneous preterm birth.

	Crude[a]			Adjusted[b]		
Analyte	Spontaneous preterm [*n*]	Term [*n*]	OR (95% CI)	Spontaneous preterm [*n*]	Term [*n*]	OR (95% CI)
IQR GLY[c]						
Visits 1 and 3 average	23	103	1.67 (0.97, 2.88)	20	97	1.44 (0.73, 2.85)
Visit 1 (18 ± 2 wk)	23	142	1.15 (0.72, 1.85)	20	135	1.34 (0.82, 2.20)
Visit 3 (26 ± 2 wk)	35	155	1.49 (1.09, 2.05)	32	148	1.45 (1.00, 2.10)
IQR AMPA[d]						
Visits 1 and 3 average	23	103	1.59 (1.10, 2.30)	20	97	1.52 (0.98, 2.35)
Visit 1 (18 ± 2 wk)	23	142	0.94 (0.70, 1.28)	20	135	0.98 (0.74, 1.29)
Visit 3 (26 ± 2 wk)	35	155	1.76 (1.31, 2.36)	32	148	1.88 (1.35, 2.62)

Note: AMPA, aminomethylphosphonic acid; BMI, body mass index; CI, confidence interval; GLY, glyphosate; IQR, interquartile range; OR, odds ratio.
[a]Biomarkers are adjusted for specific gravity using the formula: $GA_{SG} = GA[(1.019–1)/(SG–1)]$, where GA_{SG} is the SG-adjusted GLY or AMPA concentration (μg/L); GA is the observed GLY or AMPA concentration; 1.019 is the population median specific gravity; and SG is the specific gravity of the urine sample.
[b]Models are adjusted for maternal age, education, prepregnancy BMI, and smoking.
[c]IQRs were calculated based on GLY distribution in controls. IQRs were 0.56, 0.52, and 0.54 for Visit 1, Visit 3, and the Visits 1 and 3 average, respectively.
[d]IQRs were calculated based on AMPA distribution in controls. IQRs were 0.27, 0.40, and 0.30 for Visit 1, Visit 3, and the Visits 1 and 3 average, respectively.

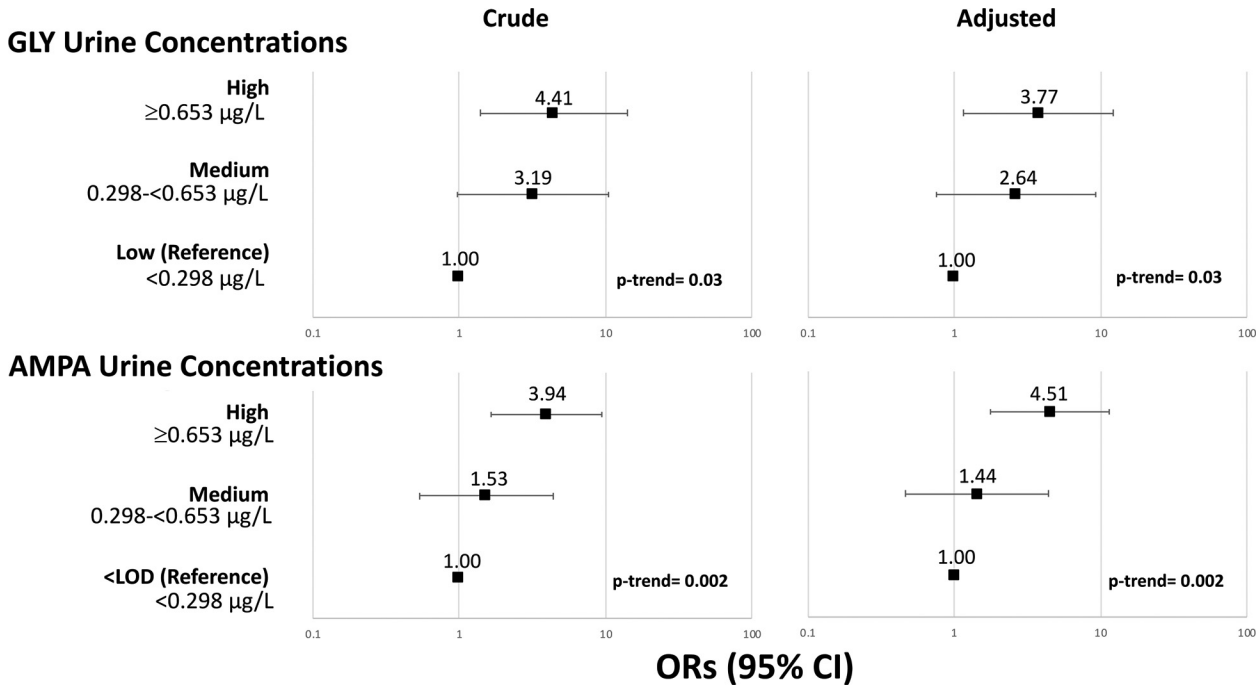

Figure 2. Odds ratios by level of urinary GLY and AMPA concentrations measured in maternal urine at Visit 3 (26 ± 2 wk gestation) and spontaneous preterm birth. Corresponding numeric data for Visit 1, Visit 3, and the pregnancy average are provided in Table S3. Biomarkers are adjusted for specific gravity using the formula: $GA_{SG} = GA[(1.019–1)/(SG–1)]$, where GA_{SG} is the SG-adjusted GLY or AMPA concentration (µg/L); GA is the observed GLY or AMPA concentration; 1.019 is the population median specific gravity; and SG is the specific gravity of the urine sample; $n = 208$; 35 cases, 155 controls. Adjusted models are adjusted for maternal age, education, prepregnancy BMI, and smoking; $n = 180$; 32 cases, 148 controls. The *x*-axes are shown on a log-10 scale. Note: AMPA, aminomethylphosphonic acid; BMI, body mass index; CI, confidence interval; GLY, glyphosate; OR, odds ratio.

AMPA in controls are similar to those of the study by McGuire et al. (2016) for Visit 3 (range: ≤0.20 to 1.52 µg/L) but higher for Visit 1 (range: ≤0.20 to 10.08 µg/L).

To our knowledge, only one other study has examined urinary GLY during pregnancy and length of gestation in humans (Parvez et al. 2018). Parvez et al. (2018) reported that urinary GLY, measured in pregnant women from rural Indiana ($n = 71$) was significantly correlated with shortened gestational length. To our knowledge, no other study to date has examined the effect of AMPA on either PTB or length of gestation.

We report consistent findings of increased odds of PTB with higher GLY and AMPA exposure biomarkers at Visit 3 but not Visit 1 or the visit average. It is unclear if this shows a potentially increased susceptibility to GLY exposures occurring later in pregnancy or if it was an artifact because Visit 3 had the largest sample size. We compared demographics across the three study groups to look for any notable differences between them (Table S6). A visual examination of characteristics across groups did not reveal any major differences between them. Further visual analysis of potential differences between cases with Visit 1 samples vs. those with only Visit 3 samples revealed that cases with only Visit 3 samples may have been more likely to be single, have a household income of <USD10,000, been never-alcohol drinkers, and been more likely to have had a prior PTB, although the number of cases with Visit 3 samples only was small ($n = 18$), limiting our ability to make robust comparisons. Future studies should also endeavor to have multiple time points, which would help address the temporal variability of GLY and provide further insight into potential windows of susceptibility to GLY or AMPA exposures during pregnancy.

One mechanism by which GLY may contribute to the etiology of PTB is by inducing oxidative stress. Toxicology research in multiple tissues and systems implicates oxidative stress as a potential mechanism for GLY toxicity in nontarget species. For example, recent studies report GLY-induced oxidative stress and apoptosis in maturing mouse oocytes (Zhang et al. 2019), markers of oxidative stress in chicks of breeder hens that had been exposed to GLY (Fathi et al. 2019), and the protective effects of the antioxidant, resveratrol, against GLY-induced oxidative stress, lipid peroxidation, and damage in rat brain, heart, liver and renal tissues (Turkmen et al. 2019). Epidemiological studies similarly report increased oxidative stress biomarkers in pregnant women who go on to deliver preterm or have shortened gestation lengths (Ferguson et al. 2015; Longini et al. 2007; Stein et al. 2008). We have also recently reported associations between oxidative stress and PTB in the PROTECT cohort. Markers of oxidative stress, 8-iso-prostaglandin $F2_{\alpha}$, and its primary metabolite, prostaglandin $F2_{\alpha}$, measured in the urine, were significantly associated with increased odds of PTB (Eick et al. 2020). Unfortunately, little is known about any potential relationships between AMPA and oxidative stress. We will pursue further research into potential associations between GLY, AMPA, oxidative stress, and PTB in the PROTECT cohort.

The present study is limited in several ways. In addition to being an environmental degradate of GLY, AMPA is a breakdown product for amino polyphosphonate chemicals, which may be used as detergents, antiscaling agents, and fire retardants (Grandcoin et al. 2017). The respective contributions of these various sources to overall AMPA exposure in the environment are not well understood (Struger et al. 2015) and difficult to determine owing to the lack of a reliable analytical methodology (Studnik et al. 2015); therefore, we cannot know what portion of the AMPA measured here may have originated from GLY and what portion may have come from one of these alternative sources. In our study, GLY and AMPA were significantly correlated ($\rho = 0.43$ and 0.51, for Visits 1 and 3, respectively), indicating

that at least some of the AMPA source was likely to be GLY. We were unable to find any studies examining specific sources of human exposure to GLY or AMPA in Puerto Rico. In addition, the relatively short half-life of GLY may have limited our ability to address the temporal variability of exposure during pregnancy; thus, we may have missed some exposure at sensitive developmental stages, even with two time points. Although larger than the only previous human study of prenatal GLY exposure and length of gestation, our sample size was still relatively modest. Our modest sample size likely influenced the relative precision of our estimates, particularly for Visit 1 and the visit average, where there were fewer observations, and for tertile analyses, analyses stratified by infant sex, and those limited to the spontaneous preterm subtype. A larger sample size would have allowed a more detailed assessment of dose–response relationships and alternative outcomes and a more robust assessment of differences by infant sex. Similar studies should be explored in other populations, preferably with larger sample sizes. Our study sample included all PTB cases recorded to date with available urine samples at Visit 1 or Visit 3, as well as randomly selected controls who also had Visit 1 or Visit 3 urine samples available. By chance, all PTB cases had Visit 3 urine samples, and a subset of these also had a sample for Visit 1; there were no cases that had only a Visit 1 sample (case urine samples = 35, 53, and 35 for Visit 1, Visit 3, and both visits, respectively). This differed from controls, where sample availability was more evenly distributed (142 control urine samples = 142, 155, and 103 for Visit 1, Visit 3, and both visits, respectively). These unintentional differences in selection of cases and controls, as a result of differing sample availability, may have increased the potential for bias or spurious differences in associations for GLY and AMPA by study visit. It is also important to acknowledge the potential for bias due to uncontrolled confounding by other pesticides or contaminants or by other factors not measured here. We attempted to address some of the potential for this by examining MBP and MiBP as potential confounders. Missing data was also a problem for two potential confounders of interest—household income and prior PTB—although we attempted to address this in a sensitivity analysis. Finally, the PROTECT cohort is restricted to healthy pregnant women without any preexisting conditions known to be associated with poor pregnancy outcomes. Although this allowed us to examine associations between GLY and AMPA and PTB without potential confounding by known comorbidities, it may somewhat limit the generalizability of the findings.

Despite its limitations, this is only the second study, and the largest to date, to directly measure prenatal GLY in maternal urine and its associations with pregnancy outcomes. It is also the first pregnancy study to measure both GLY and its environmental breakdown product, AMPA. Detection of AMPA in urine samples from pregnant women, and evidence of its association with PTB, supports the need for further investigation of AMPA because AMPA is a major environmental degradate of GLY, as well as other amino polyphosphonate chemicals; it is quite persistent in the environment; and there is little to no information about its potential effects on human health. Additional research is also needed to clarify the sources of AMPA in the environment and to determine how to reduce or prevent exposures. To our knowledge, this is the first study to examine the effect of AMPA on PTB or length of gestation. In addition, the availability of two measurements for the majority of participants provided a more stable estimate of GLY and AMPA exposure during pregnancy and allowed us to begin exploring windows of susceptibility, which is important because GLY has a relatively short biological half-life. Another strength of this study is that we were able to look at the spontaneous subtype of PTB, which may provide some insight into potential mechanisms. Previous work suggests that the association between environmental toxicants and PTB may be driven by spontaneous preterm births (Ferguson et al. 2014).

This study provides evidence for associations between urinary concentrations of GLY and its environmental degradate, AMPA, measured around the 26th week of pregnancy, and increased odds of PTB. Given the widespread use of GLY, multiple potential sources of AMPA, and AMPA's persistence in the environment, as well as the potential for long-term adverse health effects in preterm infants, further investigation in PROTECT and other populations is warranted.

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